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Oxidative stress, which results from an imbalance between ROS and antioxidant capacities, can cause a wide range of direct or indirect DNA damage. There are extensive DNA repair systems that can correct DNA damage caused by ROS before cell replication and mutation fixation. Although oxidative stress appears to be important in the etiology of prostate cancer, so far there is no study to comprehensively investigate the association between DRC of oxidative DNA damage as a phenotype and prostate cancer risk. We hypothesize that DRC of oxidative DNA damage as a phenotype may modify prostate cancer risk. We have completed the analysis of DRC in 350 prostate cancer patients and 350 healthy controls. Overall, we didn't observe any significant association between DRC and prostate cancer risk. Therefore, we have concluded that DRC is not a risk factor for prostate cancer

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Table of Contents

	Page
Introduction……..…..	**4**
Body……..	**4**
Key Research Accomplishments…….…….	**9**
Reportable Outcomes……	**10**
Conclusion……	**10**

Introduction

Many of the known and suspected risk factors for prostate cancer are associated with elevated levels of reactive oxygen species (ROS) (advancing age, inflammation, androgen, high-fat diet), or decreased antioxidant capabilities (fruit and vegetable consumption, specific dietary antioxidants, such as selenium, vitamin E and carotenoids). Oxidative stress, which results from an imbalance between ROS and antioxidant capacities, can cause a wide range of direct or indirect DNA damage. There are extensive DNA repair systems that can correct DNA damage caused by ROS before cell replication and mutation fixation. For instance, ROS-caused base damages and single strand breaks are mainly repaired by base excision repair (BER) and nucleotide excision repair (NER); DNA adducts caused by ROS-induced lipid peroxidation are repaired by NER; and ROS caused-DNA double strand breaks are repaired by homologous recombination repair (HRR) and non-homologous end-joining (NHEJ). However, DNA repair capacity (DRC) is substantially variable among individuals in the population, and suboptimal DRC of oxidative DNA damage might increase genomic instability and hence, increase risk of cancer. Although oxidative stress appears to be important in the etiology of prostate cancer, so far there is no study to comprehensively investigate the association between DRC of oxidative DNA damage as a phenotype and prostate cancer risk.

Body

Study subject recruitment: At the end of study, we have recruited 350 prostate cancer patients and 350 healthy controls. The number is over what we proposed in the grant: 300 cases and 300 controls. Therefore, we are very successful in study subject recruitment. The basic demographic characteristics among cases and controls and clinical characteristics in cases are presented in Table 1. Briefly, the mean age of cancer diagnosis is 67.9 years old for the cases and the mean age of entering the study is 67.5 years old for the controls. In terms of cigarette smoking habit, 48.8% of the cases and 50.2% of the controls are never smokers. Forty-three percent of the cases and 42% of the controls are former smokers. About 8.2% of the cases and 7.8% of the controls are current smokers. In terms of the body mass index (BMI), more than half of the cases and controls are less than 25 (58% for the cases and 60.1% for the controls). About 38.8% of the cases and 36.*% of the controls, their BMI is between 25 and 29. For the gleason score in the cases, 58.3% of the cases have gleason score between 4 and 6 and 21.5% of the cases have gleason score at or above 7. The median PSA levels are 7.4 for the cases. For the clinical stage, most of the cases (83.9%) are in T1/T2.

Study participants provided a 30 ml non-fasting blood specimen, which was drawn at the phlebotomy service at Roswell Park Cancer Institute (RPCI). Blood specimens were immediately transported to the DataBank and BioRepositories (DBBR) laboratory for processing, logged by a unique barcode identification number, and banked in 0.5 ml straws (serum, red blood cells, plasma, buffy coats, and whole blood for DNA). For this study, we received 10 ml of each participant's blood, which were processed and aliquoted for all proposed molecular assays. Peripheral blood lymphocytes (PBLs) were isolated

from the whole blood by standard techniques using Ficoll-Hapaque and then frozen for the measurement of DRC. Aliquots of the isolated lymphocytes were placed in small vials with $4x10^6$ cells in each vial; frozen in a freezing medium containing 50% fetal bovine serum (FBS) (Invitrogen), 40% of RPMI 1640 (Invitrogen) and 10% of DMSO (Sigma); and stored in a liquid nitrogen tank.

Table 1. Distribution of characteristics among cases and controls and clinical characteristics at diagnosis (cases)

	Cases (n = 350)	Controls (n = 350)	
Characteristics at baseline	N (%) or mean (SD)	N (%) or mean (SD)	P
Age	67.9 (8.4)	67.5 (8.4)	0.74
Cigarette smoking status			
Never	171 (48.8)	175 (50.2)	0.86
Former	150 (43.0)	147 (42.0)	
Current	29 (8.2)	28 (7.8)	
BMI, kg/m^2			
<25	203 (58.0)	210 (60.1)	0.67
25–29	136 (38.8)	129 (36.8)	
30+	11 (3.2)	11 (3.2)	
Gleason score at diagnosis			
4–6	204(58.3)	—	
3 -4	64 (18.2)		
7	28 (8.0)	—	
8–10	54 (13.5)	—	
missing	7 (2.0)	—	
PSA at diagnosis, ng/mL, median (IQR)	7.3 (5.1–12.7)	—	
Clinical stagee at diagnosis			
T1/T2, NX/NO	294 (83.9)	—	
T3, NX/NO	17 (4.9)	—	
T4 or N1 or M1	21 (6.1)	—	
Missing	18 (5.1)	—	

Specific aim 1: *we will measure levels of 8-OH-dG after exposure to H_2O_2 in PBLs in 300 men with prostate cancer and 300 healthy controls, using ELISA based mutagen sensitivity assay.* Our hypothesis is that cases will exhibit higher levels of 8-OH-dG after exposure to H_2O_2 (reflecting lower BER activity) compared with healthy controls. At the end of study, the proposed 8-OH-dG analysis has been carried out in 350 prostate cancer cases and 350 healthy controls.

8-OH-dG analysis method: The 8-OH-dG assay is a modified mutagen sensitivity assay to assess BER capacity. In brief, for each sample, $2x10^6$ PHA-stimulated lymphocyte cultures were established using 5ml of RPMI 1640 medium supplemented with 15% FBS. Cell cultures were established for 72 hours prior to the mutagen treatment. Separate cultures for each study subject were set up to measure baseline levels

of 8-OH-dG (not treated with H_2O_2). We have determined that 10ul of 0.1% H_2O_2 added 1 hour prior to cell harvesting is sufficient to induce oxidative DNA damage at nuclear, provide enough time to repair oxidative DNA damage, but is not cytotoxic. After treatment, both H_2O_2 treated lymphocytes and negative controls were harvested. Genomic DNAs were extracted by using QIAamp DNA Blood Mini Kit (Qiagen). Then, ELISA based mutagen sensitivity assay were performed for both H_2O_2 treated lymphocytes and negative controls. HT 8-OH-dG ELISA kits from Trevigen (http://www.trevigen.com/item/5/24/0/426/HT_8oxodG_ELISA/) were used in the study to quantify the levels of 8-OH-dG. The instruction from the kit was strictly followed. For each subject, the difference of levels of 8-OH-dG between H_2O_2 treated lymphocytes and negative controls were calculated and deemed as the index of sensitivity to H_2O_2. Because BER is the major DNA repair pathway for 8-OH-dG base damage, the index of sensitivity to H_2O_2 also reflects the capacity of BER.

Results: The mean levels of 8-OH-dG were higher in cases than in controls (4.13 vs. 3.57, P=0.42), although the difference didn't reach statistical significance (Table 2). In further stratified analysis, using 75% levels of 8-OH-dG in controls as the cutoff point, we found higher levels of 8-OH-dG was not associated with prostate cancer risk (OR= 1.42, 95% CI: 0.87 to 2.08) after adjusting age, BMI, gleason score, PSA, smoking status, and clinical stage. The association is inconsistent with what we found in previous annual report. The difference might be due to the sample size difference.

Table 2. Levels of 8-OH-dG and prostate cancer risk

	Cases, mean (SD)	Controls, mean (SD)	P value
Overall	4.13 (0.46)	3.57 (0.55)	0.42
DRC (%)	Cases n (%)	Controls n (%)	Adjusted OR (95% CI)
75th percentile in controls as cutoff point			
≥3.24	114 (13.5)	87 (24.7)	Reference
<3.24	244 (86.5)	263 (75.3)	1.42 (0.87-0.28)

Specific aim 2: *we will assess levels of DRC of DNA adducts induced by 4-HNE in PBLs in 300 prostate cancer cases and 300 healthy controls, using plasmid based modified HCR assay*. 4-HNE is a major product of endogenous lipid peroxidation. 4-HNE caused DNA adducts is mainly repaired by NER. Our hypothesis is that cases will exhibit lower levels of NER of 4-HNE caused DNA adducts compared with healthy controls. The proposed 4-HNE based host cell reactivation (HCR) assay has been carried out in 350 prostate cancer cases and 350 healthy controls.

4-HNE DNA repair analysis: 4-HNE assay is a modified host cell reactivation (HCR) assay to assess NER capacity of 4-HNE caused DNA adducts on reporter plasmid. Plasmid pGL-3 luciferase reporter was modified with 4-HNE (Caymen) by standard methods. In brief, a stock solution was prepared by dissolving 4-HNE in methanol at a concentration of 100 mg/mL. The purified plasmid DNA was dissolved in TE buffer, mixed with a 4-HNE solution, and incubated at 37°C for 30 hours. Control plasmids were treated with methanol only. The unreacted 4-HNE was removed by repeated phenol and

diethyl ether extractions, and the treated plasmids were then ethanol precipitated and dissolved in TE buffer. We have demonstrated that under these modification conditions the 4-HNE-dG adduct is the major DNA adduct that is formed. 2.0 x 10^6 lymphocytes were cultured in RPMI 1640 supplemented with 20% FCS and PHA at 37°C and 5% CO_2 and incubated for 72 hours. The DEAE-dextran (Pharmacia Biotech) method was used to transfect aliquots with undamaged pGL-3 and pGL-3 damaged by 4-HNE. The cultures were then incubated for 40 hours before harvesting. After that, the cell pellets were suspended in reporter lysis buffer (Promega), frozen, and thawed once in ethanol-dry ice and 37°C water baths and then centrifuged. Luciferase (LUC) assay was applied to quantify the activity of luciferase. For each LUC assay, 20 µL of cell extract supernatant was mixed with 100 µL of Luciferase Assay Substrate (Promega) in a tube at room temperature. LUC activity in arbitrary light-intensity units was measured with a luminometer (Turner). LUC light-intensity units were recorded for the cells with undamaged plasmids (control) and 4-HNE–damaged (repair) plasmids. DNA repair capacity (%) was calculated as the product of 100% and the ratio of the damaged plasmid values to the undamaged plasmid values.

Results: The mean levels of 4-HNE based HCR were marginally lower in cases than in controls (7.3% vs. 8.4%, P=0.67) (Table 3), although the difference didn't reach statistical significance. In further stratified analysis, using 25% levels of 4-HNE based in controls as the cutoff point, we found lower levels of 4-HNE based was not associated with the prostate cancer risk (OR= 1.21, 95% CI: 0.74 to 1.79) after adjusting age, BMI, gleason score, PSA, smoking status, and clinical stage. The association is consistent with what we found in previous annual report.

Table 3. Levels of HCR and prostate cancer risk

	Cases, mean (SD)	Controls, mean (SD)	P value
Overall	7.3% (1.4%)	8.4% (2.1%)	0.67
DRC (%)	Cases n (%)	Controls n (%)	Adjusted OR (95% CI)
25th percentile in controls as cutoff point			
<5.8	100 (28.5)	88 (25.1)	Reference
≥5.8	250 (71.5)	262 (74.9)	1.21 (0.74 – 1.79)

Specific aim 3: *we will assess levels of HHR and NHEJ of double strand breaks in PBLs in 300 men with prostate cancer and 300 healthy controls, using plasmid based modified HCR assays.* Our hypothesis is that cases will exhibit lower levels of HR and NHEJ compared with healthy controls. For HR assay, the assay has been carried out in 350 prostate cancer cases and 350 healthy controls.

HRR assay: HRR assay is a modified HCR assay to specifically assess HRR capacity. In brief, we constructed two plasmids, pGL-luc-del1 and pGL-luc-del2, by deleting part of Luc report gene in PGL-3-luc vector. There is about 400bp homolog sequence left between 2 deletions. Because of the deletion, none of the plasmids can produce normal luciferase protein. Both plasmids were co-transfected into cells and cultured 48 hours. If the cells have normal HRR ability, a normal pGL-3-luc plasmid,

which can synthesize luciferase, were generated by HRR. Therefore, the luciferase activity is an index for HRR ability. Methods for transfection and luciferase detection are similar to those used in 4-HNE assay described above. Briefly, the DEAE-dextran (Pharmacia Biotech) method was used to transfect aliquots with pGL-luc-del1 and pGL-luc-del2 vs pGL-luc plasmids. The cultures were then incubated for 40 hours before harvesting. After that, the cell pellets were suspended in reporter lysis buffer (Promega), frozen, and thawed once in ethanol-dry ice and 37°C water baths and then centrifuged. Luciferase (LUC) assay was applied to quantify the activity of luciferase. For each LUC assay, 20 µL of cell extract supernatant was mixed with 100 µL of Luciferase Assay Substrate (Promega) in a tube at room temperature. LUC activity in arbitrary light-intensity units was measured with a luminometer (Turner). LUC light-intensity units were recorded for the cells with pGL-luc-del1 and pGL-luc-del2 plasmids vs normal pGL-luc plasmid. DNA repair capacity (%) was calculated as the product of 100% and the ratio of the damaged plasmid values to the undamaged plasmid values.

NHEJ assay: NHEJ assay is a modified HCR assay to specifically assess NHEJ capacity. The plasmid pGL3-MCS as a substrate for end-joining–mediated recircularization was derived from pGL3-Control. To introduce additional unique restriction cleavage sites between the promoter and the luciferase gene, part of the multiple cloning site of pcDNA3.1 (Invitrogen) was amplified by PCR. This PCR fragment was inserted into the unique HindIII site of pGL3-Control. Blunt-ended double strand breaks were created by EcoRV cleavage. The linear plasmid doesn't have the ability to produce luciferase protein. The linear plasmid will be transfected into cells and cultured 48 hours. If the cells have normal NHEJ ability, the linear plasmid will be rejoined and produce a normal pGL-3-luc plasmid which can synthesize luciferase protein. Therefore, the luciferase activity is an index for NHEJ ability. Methods for transfection and luciferase detection are similar to those used in 4-HNE assay described above. In brief, the DEAE-dextran (Pharmacia Biotech) method was used to transfect aliquots with linear pGL-3-luc plasmid vs normal circular pGL-3-luc plasmid. The cultures were then incubated for 40 hours before harvesting. After that, the cell pellets were suspended in reporter lysis buffer (Promega), frozen, and thawed once in ethanol-dry ice and 37°C water baths and then centrifuged. Luciferase (LUC) assay was applied to quantify the activity of luciferase. For each LUC assay, 20 µL of cell extract supernatant was mixed with 100 µL of Luciferase Assay Substrate (Promega) in a tube at room temperature. LUC activity in arbitrary light-intensity units was measured with a luminometer (Turner). LUC light-intensity units were recorded for the cells with circular plasmids (control) and linear (repair) plasmids. DNA repair capacity (%) was calculated as the product of 100% and the ratio of the linear plasmid values to the circular plasmid values.

Results: The mean levels of HHR activity were lower in cases than in controls (10.5% vs. 11.7%, P=0.47) (Table 4), but the difference didn't reach statistically significant. In further stratified analysis, using 25% levels of HHR based in controls as the cutoff point, we found lower levels of HHR based was not associated with the prostate cancer risk (OR= 1.30, 95% CI: 0.72 to 1.85) after adjusting age, BMI, gleason score, PSA, smoking status, and clinical stage.

The mean levels of NHEJ activity were lower in cases than in controls (8.1% vs. 8.8%, P=0.57), but the difference didn't reach statistically significant. In further stratified analysis, using 25% levels of HHR based in controls as the cutoff point, we found lower levels of NHEJ based was not associated with the prostate cancer risk (OR= 1.11, 95% CI: 0.64 to 2.06) after adjusting age, BMI, gleason score, PSA, smoking status, and clinical stage. The association is consistent with what we found in previous annual report.

Table 4. Levels of HHR and NHEJ and prostate cancer risk			
	Cases, mean (SD)	Controls, mean (SD)	P value
HHR			
Overall	10.5% (3.6%)	11.7% (4.1%)	0.47
DRC (%)	Cases n (%)	Controls n (%)	Adjusted OR (95% CI)
25th percentile in controls as cutoff point			
<6.4	107 (28.5)	88 (25.1)	Reference
≥6.4	243 (71.5)	262 (74.9)	1.30 (0.72 – 1.85)
NHEJ			
Overall	8.1% (2.3%)	8.8% (2.6%)	0.57
DRC (%)	Cases n (%)	Controls n (%)	Adjusted OR (95% CI)
25th percentile in controls as cutoff point			
<5.8	96 (28.5)	89 (25.4)	Reference
≥5.8	254 (71.5)	261 (74.6)	1.11 (0.64 – 2.06)

Overall, we didn't observe any significant association between DNA repair capacity and prostate cancer risk.

Key Research Accomplishments

- Prostate cancer cases don't exhibit higher levels of 8-OH-dG after exposure to H_2O_2 (reflecting lower BER activity) compared with healthy controls.
- Prostate cancer cases don't exhibit lower levels of NER of 4-HNE caused DNA adducts compared with healthy controls.
- Prostate cancer cases don't exhibit lower levels of HR compared with healthy controls.
- Prostate cancer cases don't exhibit lower levels of NHEJ compared with healthy controls.

- In training, Dr. Zhao has involved in Dr. Mohler's SPORE grant application.

Reportable outcomes

- Oral presentation: *Leonardo Medico, Hua Zhao, "Oxidative Stress, DNA Repair and Prostate Cancer Risk"* at DOD IMPACT meeting, March, 2011.

- Poster presentation: *Leonardo Medico, Hua Zhao, "Oxidative Stress, DNA Repair and Prostate Cancer Risk"* at DOD IMPACT meeting, March, 2011.

- We are working on a manuscript right now.

Conclusion

To our knowledge, this is the first study well-designed specifically, comprehensively and sufficiently powered to elucidate the role of DRC of oxidative DNA damage in prostate cancer development. To address the question, we have applied four different novel phenotypic assays, which give us power to measure effects of whole pathway. These four different DRC phenotypic assays specifically assessed DRC of oxidative DNA damage in four different pathways. Unfortunately, we didn't observe any significant association between DRC of oxidative DNA damage and prostate cancer risk. Specifically, higher levels of 8-OH-dG, which reflect lower BER, were not associated with prostate cancer risk. Lower levels of 4-HNE DNA damage repair were not associated with prostate cancer risk. Lower levels of HHR and NHEJ were not associated with prostate cancer risk. Our results cast the doubt of the roles of DNA repair capacity in the etiology of prostate cancer. Further analysis is needed, especially with large sample size.

One possibility of the negative association between DNA repair capacity and prostate cancer risk might be due to the fact of using surrogate tissues, not the target tissues. In this study, we have used lymphocytes as a surrogate tissue. Although studies have shown the correlations of DNA damage between lymphocytes and several other tissues, the exact correlations between lymphocytes and prostate tissues are unknown so far. Because DNA damage levels tend to be smaller compared to target tissues, measuring DNA damage in lymphocytes might bias the association to null. This is what happened in our study. We have observed the difference between prostate cancer cases and healthy controls for all DNA repair assays. However, none of the assays shows significant results. Another possibility is the sample size. Although 350 cases and 350 controls are not small for DNA repair assays, it might be small to detect the modest to small effect size.

Nevertheless, our study has provided a unique opportunity to comprehensively answer, for the first time, whether suboptimal DRC of oxidative DNA damage is a predictor of prostate cancer. The study helps understand the genetic events leading to the

development of prostate cancer and explore the genetic basis linking oxidative stress and prostate cancer.